'Non-specific' binding

The problem, and a solution

Carl M. MENDEL* and Dirk B. MENDEL†
*Cardiovascular Research Institute, University of California, San Francisco, CA 94143, U.S.A., and
†Department of Physiology, Dartmouth Medical School, Hanover, NH 03756, U.S.A.*

The concept of 'non-specific' binding, as it relates to studies of the binding of hormones to their receptors, is reviewed. It is concluded that the most widely used operational definition, namely binding that is not displaceable by an excess of unlabelled ligand, is often inaccurate, resulting either in overestimation of the number of high-affinity receptors and underestimation of the affinity of a given hormone for its receptor, or in a curvilinear Scatchard plot suggesting (artifactually) the presence of negative co-operativity or multiple classes of binding sites. The general use of an alternative approach to non-specific binding, in which the non-specific component is assessed from an analysis of total binding, is advocated. The superiority of this approach is illustrated with data on the binding of high-density lipoproteins to their receptors.

Some 'non-specific' binding, i.e. binding to impurities in the assay, is observed in most studies of the binding of labelled hormones to their receptors. The early assumption that non-specific binding was unsaturable (and therefore non-displaceable by competing ligands) permitted its first operational definition: binding that was not displaceable by excess concentrations of ligands that bound to the physiological receptor. 'Specific' binding to the receptor was then calculated by subtracting the measured non-specific binding from the measured total binding.

Such non-displaceable binding is probably still the most widely used operational definition of non-specific binding. It is now clear, however, that the assumption on which this operational definition is based is incorrect: that is, non-specific binding is saturable. Its saturability has been demonstrated by three findings: (1) the binding of labelled hormones to biological membranes devoid of receptors and to inert materials (models of non-specific binding) is displaceable (Cuatrecasas & Hollenberg, 1975; Phillips, 1976; Dana *et al.*, 1977; Mendel & Almon, 1979); (2) the relationship between measured non-specific binding and free ligand concentration is not linear when studied over a wide enough range of free ligand concentration (at least 100-fold), but instead can be

described (empirically) by a power function ($y = ax^b$) with an exponent less than 1 (Almon & Appel, 1976; Mendel & Almon, 1979); (3) when increasing concentrations of competing ligand are used to define non-specific binding (e.g. a concentration 1000-fold above that required to saturate 90% of the receptors, as opposed to 100-fold), the amount of measured non-specific binding decreases (thereby increasing the amount of calculated binding to the receptor) (Munck, 1976; Mendel & Almon, 1979).

The recognition that non-specific binding is saturable, a finding that theoretically invalidates such non-displaceable binding as an appropriate measure of non-specific binding, has not yet led to a thorough re-evaluation of its operational definitions. We have therefore undertaken such a re-evaluation; we show that non-displaceability as a measure of non-specific binding is operationally (as well as theoretically) often not valid, and propose the general use of an alternative approach to non-specific binding.

Analysis and discussion

Although non-specific binding probably consists of numerous types of associations with numerous classes of sites, for the sake of this analysis we treat it as binding to a set of identical and independent sites that have affinity and capacity for the

* To whom correspondence should be addressed.

hormone being studied very different from those of the receptor (a more complex formulation would not qualitatively alter our findings). We do not consider whether non-specific binding may ever have physiological relevance, but simply think of it as binding to sites other than the receptor being studied.

The binding of a ligand to two classes of non-interacting sites (in this case the receptor and the non-specific site) is described by the equation:

$$B = n_1 F/(K_{D1}+F)+n_2 F/(K_{D2}+F) \qquad (1)$$

where B is the concentration of bound ligand, n_1 and n_2 are the concentrations of the two classes of binding sites, F is the concentration of free ligand, and K_{D1} and K_{D2} are the dissociation constants of the ligand for each of the classes of sites. When $K_{D2} \gg F$, this equation reduces to:

$$B = n_1 F/(K_{D1}+F)+n_2 F/K_{D2}$$

and the binding to the second class of sites appears to be described by a linear function (i.e. it appears to be unsaturable). Many cases of non-specific binding can probably be empirically described by such a linear function when the concentration of free ligand is in the range required to study the receptor. When an excess concentration of un-labelled ligand is added to the system, however (i.e. in an attempt to measure the non-specific binding), F may approach K_{D2}; then the non-specific binding will no longer appear linear, but instead will be seen as saturable (and therefore displaceable).

The types of errors that occur when non-displaceable binding is used as a measure of non-specific binding are apparent from these considerations. An example will clarify them. Consider a system with 100 identical and independent receptors, and a ligand (in labelled and unlabelled form) that binds to these receptors with a K_D of 1 nM. Consider also that, when the free ligand concentration in this system is 1 nM, about 20% of the measured total binding is non-specific. Such non-specific binding could reasonably result from binding of the labelled ligand to a set of identical and independent sites with a 1000-fold lower affinity for the ligand ($K_D = 1 \mu$M) and a 100-fold higher capacity ($n = 10000$) than those of the receptors, and so consider that this affinity and capacity define the non-specific binding. (Also, consider that the concentration of binding sites is low relative to the concentration of total ligand, so that the concentration of total ligand is approximately the same as that of free ligand.) Now, if we employ the usual operational definition of non-specific binding in this system and define it as binding of the labelled ligand that is not displace-

able by a concentration of the same (unlabelled) ligand 100-fold greater than that required to saturate 90% of the receptors (1 μM), the problem becomes readily apparent. From eqn. (1) it can be seen that, when the concentration of the free labelled ligand is at its K_D for the receptor (1 nM), 50 receptors and ten non-specific sites are occupied at equilibrium. But, when the unlabelled ligand is then added, the total (labelled plus unlabelled) free ligand concentration is 1 μM and half (5000) the non-specific sites are occupied, five by the labelled ligand (1/1000, i.e. 1 nM/1 μM, of the total ligand). These five sites are correctly defined as non-specific because the labelled ligand is seen as being non-displaceable from them. However, since there are really ten non-specific sites occupied at a free ligand concentration of 1 nM, five of these are defined as receptors. The overestimation of the amount of specific binding to the receptors at a free ligand concentration of 1 nM (its K_D), then, is 10% (5/50). Similar analyses of this operational definition of non-specific binding at concentrations of free labelled ligand of 0.1 nM and 10 nM yield an overestimation of specific binding in each of these cases of 5.6% and 54% respectively (Fig. 1).

Thus the problem with using non-displaceability as an operational definition of non-specific binding is that it can result in overestimation of the amount of binding to receptors. Furthermore, since this overestimation is not uniform throughout the binding study, but instead increases as the concentration of free labelled ligand increases, saturation is not perceived at the appropriate point (Fig. 1), and the data suggest (artifactually) negative co-operativity or multiple classes of
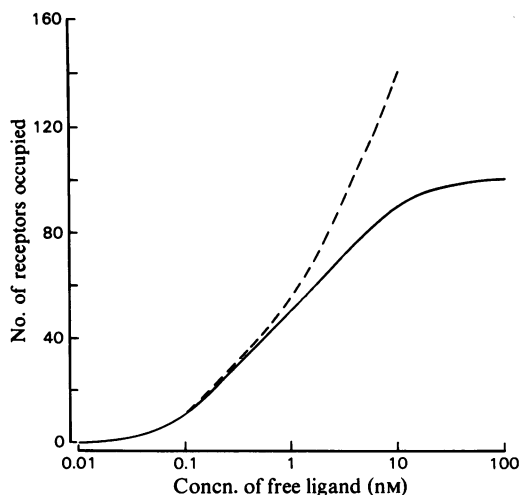

Fig. 1. *Deviation of measured specific binding (i.e. displaceable binding)* (----) *from 'true' specific binding* (——) *for the hypothetical system presented in the text*

binding sites. When depicted on a Scatchard (1949) plot, they form a concave-upwards curve. The fact that curvilinear Scatchard plots are not more prevalent in the literature could be taken as an argument that the hypothetical system presented here is not representative of most experimental non-specific binding, but we favour an alternative explanation. As Klotz (1982) pointed out, it has (unaccountably) become customary to study the binding of ligands to receptors over only a narrow range (less than 100-fold) of free ligand concentration, thus often yielding invalid estimates of the number of receptors and their affinity for the ligand (and of the presence or absence of co-operativity). In such a study, the curvilinearity in the Scatchard plot might not be recognized, since the curve is effectively truncated (see Fig. 3 as an example). The receptors might then be seen as homogeneous, but their number would still be overestimated and their affinity for the ligand

underestimated, since some of the (low-affinity) non-specific sites would be seen as receptors.

Nevertheless, it is possible that in some experimental systems such non-displaceable binding may be an accurate measure of non-specific binding. Similarly, measuring non-specific binding by other means (such as using a different unlabelled ligand to displace the labelled ligand, or removing bivalent cations from the binding assay when they are required for binding to the receptor) may, in some systems, be valid. But what sense does it make to rely on assumptions made *a priori* (namely that only binding to the receptor is affected by the particular manipulation employed) when there is no need to do so?
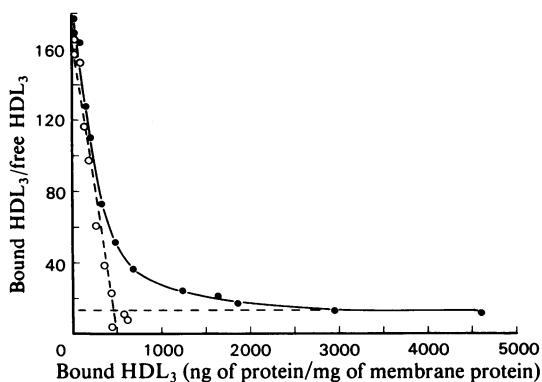

Fig. 2. *Scatchard plot of total binding of human ^{125}I-HDL$_3$ (high-density lipoproteins, density 1.125–1.21 g/cm^3) to crude plasma membranes prepared from bovine liver*
^{125}I-HDL$_3$ were incubated with membranes (0.5 mg of protein/ml) in 100 mM-NaCl/50 mM-Tris buffer, pH 7.4, containing 2% (w/v) human serum albumin for 90 min at 37°C, by which time equilibrium was attained. Bound HDL$_3$ were separated from free HDL$_3$ by vacuum filtration on Whatman GF/C glass-fibre filters that had been pre-soaked in 4% (w/v) bovine serum albumin. The free HDL$_3$ concentration ranged from 0.15 to 500 μg of protein/ml in this study. The curve is separated into two components (----) by computer analysis, as discussed in the text. The horizontal component represents non-specific binding and the more vertical component (○) represents the binding of HDL$_3$ to a single set of identical and independent binding sites ($K_D = 3$ μg of protein/ml $\approx 3 \times 10^{-8}$ M). Since a horizontal line on a Scatchard plot indicates non-saturability, it must be emphasized that the horizontal component seen here would no longer appear horizontal if data were collected at higher free ligand concentrations and plotted on an extended scale.
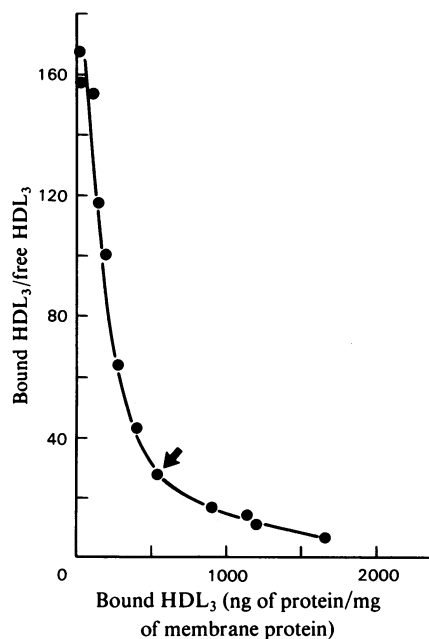

Fig. 3. *Scatchard plot of 'specific' (displaceable) binding of human ^{125}I-HDL$_3$ to crude plasma membranes prepared from bovine liver*
To obtain specific binding, binding that was not displaceable by 2 mg of unlabelled HDL$_3$/ml was subtracted from total binding at each point (this total binding is the same as that depicted in Fig. 2, except that the maximum free HDL$_3$ concentration here is 200 μg of protein/ml). The reasons that this operational definition of specific binding is not valid and that this curve is an artifact are described in the text, and this curve should be compared with the more vertical component of the curve in Fig. 2. Also of note is the fact that, if the curve were truncated at the arrow (where the free HDL$_3$ concentration is 20 μg of protein/ml, almost 7-fold above the K_D), the data would appear as a straight line.

The solution to the problem of measuring non-specific binding is that it should not be measured at all. Instead, only total binding should be measured; the appropriate equations (such as eqn. 1) can then be fitted to the data by non-linear-regression analysis. In this manner the binding data can be separated into (at least) two components, one of which represents the non-specific binding.

This approach requires no assumptions, except that the binding to the non-specific sites is described by a function other than the binding to the receptor. And it can accommodate complicated models of binding to the receptor (e.g. multiple classes of binding sites or co-operativity). Since the purpose of the present communication is to demonstrate the need for this approach, rather than to re-describe it, the reader is referred elsewhere for detailed descriptions of the methods and available computer programs (Munson & Rodbard, 1980, 1983).

The superiority of this approach to the problem of non-specific binding is illustrated by our data on the binding of high-density lipoproteins (HDL_3) to their receptors. It can be seen that by fitting eqn. (1) to the total binding data, and separating it into two components by computer analysis, we find a component that rigorously fits the expected paradigm of a ligand binding to a single class of identical and independent sites (Fig. 2). (Consideration of more complicated models of binding did not improve the fit to the data.) This finding contrasts with what is seen when an excess of unlabelled ligand is used (inappropriately) to measure non-specific binding in the same system: a curvilinear Scatchard plot results (Fig. 3), yielding incorrect information about the binding function, as predicted from our discussion.

The more general use of this approach to the problem of non-specific binding, as opposed to approaches relying on assumptions made *a priori*, should provide more accurate information on the dissociation constants, the numbers of receptors and the presence or absence of co-operativity in a variety of hormone–receptor systems.

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